NOVEL CHIMERIC PLASMINOGEN ACTIVATORS AND THEIR PHARMACEUTICAL USE

The present invention relates to recombinant chimeric proteins comprising a surfactant protein precursor N-terminally fused to a plasminogen activator or comprising a mature surfactant protein N-terminally or C-terminally fused to a plasminogen activator. The invention is also directed to the corresponding nucleic acid molecules encoding such fusion proteins as well as to a method for their production. The invention further refers to a pharmaceutical composition comprising such a fusion protein and to pharmacological uses of an inventive fusion protein for the prevention and/or treatment of inflammatory and interstitial lung diseases.

Numerous acute inflammatory and chronic interstitial lung diseases, such as the acute respiratory distress syndrome (ARDS), acute lung injury (ALI), interstitial lung disease (ILD) or idiopathic pulmonary fibrosis (IPF), are characterized by substantial surfactant abnormalities, e.g. alterations in surfactant composition, leakage of plasma proteins into the alveolar space, or intra-alveolar accumulation of fibrin (reviewed in [1, 2]).

Under these pathological conditions, the alveolar hemostatic balance is shifted towards a predominance of pro-coagulant and anti-fibrinolytic activities, whereas the fibrinolytic activity of the alveolar space is markedly reduced, with depressed levels of urokinase-plasminogen activator (u-PA; also termed urokinase), the predominant plasminogen activator in this compartment, but elevated concentrations of plasminogen activator inhibitor 1 (PAI-1) and α_2-antiplasmin [3-5]. In such a setting, fibrinogen leaking into the alveolar space due to an impaired function of the air/blood-barrier (consisting of the capillary endothelium, the interstitial space, and the alveolar epithelium) is rapidly converted into fibrin, and alveolar fibrin accumulation is observed.

The function of fibrin formation in the alveolar space is largely unknown. It may have beneficial effects in preventing pulmonary hemorrhage and serve as primary matrix of wound repair. On the other hand, alveolar fibrin may contribute to the impairment of gas exchange in acute lung injury, and a delayed clearance of alveolar fibrin may provide a provisional matrix for subsequent fibroblast invasion as well as production of extra-cellular matrix proteins and thus promote the fibroproliferative response that characterizes a prolonged course of ARDS and lung fibrosis (reviewed in [6-8]).

Pulmonary surfactant is a lipoprotein complex covering the alveolar surface of all mammalian lungs (reviewed in [9, 10]). By reducing the surface tension at the air/liquid- interface to very low levels, it makes alveolar ventilation and gas exchange feasible at low physiologic pulmonary pressures and prevents alveoli from collapsing. Pulmonary surfactant is composed of approximately 90% lipids and 10% proteins. Of the lipids, 80-90% are phospholipids, with phosphatidylcholine as the most abundant component. To date, four surfactant-associated proteins have been identified which can be divided into two groups: the hydrophilic surfactant proteins (SP) SP-A and SP-D, and the hydrophobic surfactant proteins SP-B and SP-C (reviewed in [11, 12]).

In recent years, application of exogenous surfactant preparations has become an interesting approach to restore surfactant dysfunction in pathological conditions, such as ARDS or IRDS. For example, the International PCT Application [13] discloses a pharmaceutical preparation for treating infant respiratory distress syndrome or acute lung injury, comprising at least one modification of SP-B and at least one modification of SP-C. The authors have found that by adding modifications of SP-C to pulmonary surfactant preparations containing modifications of SP-B, pharmaceutical preparations with advantageous properties are obtained. The modifications of the surfactant proteins may be recombinant proteins. U.S. Patent [14] describes a composition for pulmonary administration of a pharmaceutically active compound comprising a liposome forming compound as well as at least one alveolar surfactant protein in an amount effective to enhance transport of the liposomes across a pulmonary surface. Finally, U.S. Patent [15] describes several fragments of SP-B that exhibit surfactant activity when admixed with phospholipids. These fragments are suitable compounds for the preparation of therapeutically effective formulations for the treatment of respiratory disease.

Surfactant abnormalities are assumed to represent key events in the development of acute and chronic respiratory failure. Impairment of biophysical surfactant function with increased minimum surface tension and altered phospholipid- and surfactant protein-profiles have consistently been observed in patients with ARDS, severe pneumonia as well as interstitial lung disease (reviewed in [1, 2]). Furthermore, it has been established that alveolar fibrin formation represents the most powerful surfactant-inhibitory mechanism hitherto described. Generation of a fibrin clot in the presence of pulmonary surfactant resulted in an almost complete incorporation of hydrophobic surfactant components, such as phospholipids and the surfactant proteins SP-B and SP-C, into the nascent fibrin matrix together with severe loss of surface tension-lowering properties. In addition, surfactant-containing fibrin represents a unique structure within the

alveolar space with distinct properties. Compared to "normal" (extra-alveolar) fibrin clots, alveolar fibrin clots are characterized by altered clot architecture, altered mechanical properties and a reduced susceptibility towards proteolytic degradation (reviewed in [1]).

Thus, a correction of the hemostatic imbalance described above by increasing the alveolar fibrinolytic activity may represent a reasonable therapeutic approach to restore surfactant function. And indeed, *in vivo* and *in vitro* studies have succeeded in achieving this goal. An up-regulation of urokinase levels by adenovirus-mediated gene transfer reduced the extent of bleomycin-induced lung fibrosis in mice [16]. Furthermore, in perfused rabbit lungs urokinase treatment was followed by a pronounced improvement of gas exchange [17]. *In vitro* cleavage of surfactant-incorporating fibrin was shown to rescue surfactant material trapped in the fibrin matrix, with its surface tension-lowering properties being conserved [18, 19].

This strategy, however, might be hampered by induction of bleeding from pulmonary and extra-pulmonary sites, even if the fibrinolytic agent is primarily distributed to the alveolar space. Moreover, disadvantageous effects on surfactant function might be provoked. To overcome these limitations tools have to be developed which enhance the selectivity and potency of a fibrinolytic therapy against surfactant-containing fibrin.

Ruppert et al. have recently established such a molecular tool for targeting alveolar fibrin by chemically coupling a monoclonal anti-SP-B antibody, designated 8B5E, to human urokinase using a heterobifunctional cross-linker [20]. In another study [21], the same authors have reported chemical cross-linking of human urokinase to purified bovine SP-B. Both of these hybrid proteins were found: (1) to retain the biophysical activities compared to native SP-B, and (2) to be about 2-3 fold more effective in lysis of surfactant-containing fibrin clots and about 3-5 fold more resistant towards PAI-1 than native u-PA, thus resulting in chimeric enzymes with enhanced substrate specificity. On the other hand, due to the effort required to purify the proteins, in particular with respect to SP-B, which is purified from a natural source, to be coupled by conventional purification methods, this strategy is time-consuming and quite laborious. This disadvantage, however, may be partially overcome by the recombinant production of the two isolated proteins (e.g. urokinase and SP-B), followed by their chemical cross-linking.

For this purpose, recombinant mature SP-B might be obtained by according to U.S. Patent [22]. This patent discloses a process for producing mature alveolar SP-B using a SP-B precursor protein having a propeptide only at its N-terminus but lacking a C-terminal propeptide. In [22],

processing of the N-terminal propeptide is performed *in vitro* using a genetically engineered hydroxylamine cleavage site. This results in release of the mature peptide.

Recombinant human urokinase might be obtained in accordance with U.S. Patent [23]. Furthermore, hybrid plasminogen activators have been disclosed, e.g., in the following U.S. Patents: [24] describes a fibrinolytically active two-chain hybrid protein, wherein the chains are derived from the same or different two-chain proteases. U.S. Patent [25] describes a fibrin-specific two-chain urokinase-plasminogen activator in a therapeutic dosage form for dissolving clots *in vivo*, whereas [26] discloses the recombinant production of single-chain chimeric plasminogen activators composed of at least two subsequences of human tissue-plasminogen activator and human urokinase-plasminogen activator. The plasminogen activators disclosed in [23-26] are only for systemic application.

Another desirable feature of an efficient fibrinolytic tool for targeting alveolar fibrin would be its specificity for surfactant-containing fibrin clots. U.S. Patent [27] describes a fusion protein of lysozyme and the C-terminal propeptide of SP-B with the ten preceding amino acid residues of the mature SP-B peptide included, which is administered in a pharmaceutically acceptable medium to an individual to prevent and/or treat bacterial infections, particularly bacterial respiratory infections. By fusing lysozyme to a portion of a surfactant protein, the enzyme is delivered to the lung as the target infection site. Thus, according to [27] a SP-B fragment can be employed to target an enzymatic activity that is fused to it to a confined region of the body.

Accordingly, there is still a need for molecular tools suitable for a fibrinolytic therapy against surfactant-containing fibrin. Although the two hybrid proteins described above [20, 21] are actually functional, they have some pivotal drawbacks: First, chemical coupling requires purification of the proteins to be coupled which can be very laborious and time-consuming for its own (see above). Second in the vast majority of cases the precise composition and/or structure of the conjugate obtained is unknown due to ambiguities regarding the amino acid residues actually undergoing coupling events. Third, not every protein and every cross-linking agent are applicable to chemical coupling in a given experimental setting, and fourth the efficiencies of the coupling step may vary among experiments of the same type.

Therefore, the problem to be solved by the present inventions is to overcome these limitations and to provide a molecular tool, which not only specifically targets surfactant-containing fibrin clots

and efficiently lyses such clots but which can also be produced easily in amounts sufficient for therapeutic applications.

These goals are accomplished by a fusion protein having the features of the independent claims as well as by the method for their production. Such a fusion protein comprises:

(a) a mammalian surfactant protein precursor lacking its C-terminal propeptide, and

(b) a mammalian plasminogen activator,

wherein the surfactant protein precursor is fused at its C-terminus to the N-terminus of the plasminogen activator.

Alternatively, a fusion protein of the present invention comprises:

(a) a mature mammalian surfactant protein, and

(b) a mammalian plasminogen activator,

wherein the mature surfactant protein is fused at its C-terminus or its N-terminus to the N-terminus or the C-terminus of the plasminogen activator, respectively.

Such "single-chain" fusion proteins of the present invention (compared to the "two-chain" hybrid proteins generated by chemical coupling) appear to retain both the biophysical properties of the surfactant protein and the fibrinolytic activity of the plasminogen activator, and they are efficiently targeted to intra-alveolar surfactant-containing fibrin clots. Furthermore, the present invention provides the advantage that the subsequent purification of the nascent recombinant protein is also straightforward and can normally be performed within a day. Additionally, by employing this recombinant method it is assured that the fusion proteins are assembled in a 1:1 fashion, i.e. have a defined composition.

Considering the synthesis and processing of the surfactant proteins SP-B and SP-C *in vivo*, the apparent retention of the biophysical properties of the surfactant protein by the inventive fusion protein is particularly surprising since it contains the N-terminal propeptide of the mammalian surfactant protein. Both SP-B and SP-C are synthesized as precursor proteins by type II alveolar cells. These precursors are processed to the mature peptides during transit through the secretory pathway (reviewed in [9, 10, 12]). Due to the hydrophobicity of mature SP-B and SP-C, respectively, it is physiologically indispensable to escort them in form of precursor proteins prior to association with surfactant lipids. Otherwise, they would immediately disrupt lipid membranes, which would in turn result in cell lysis (for this reason, it has so far not been possible to produce recombinant mature SP-B in cell cultures systems such as HeLa or CHO cells).

Therefore, it must be assumed that the propeptide prevents the mature surfactant protein from exhibiting its biophysical activity during the delivery to the alveolar cells, meaning that the propeptide provides in some respect on the molecular level a "shield" against the (at that time highly detrimental) function and cell damaging properties of the mature surfactant protein. Accordingly, it was to the surprise of the inventors to find out that despite the presence of the N-terminal propeptide the fusion proteins of the invention appear to possess the biophysical properties of the mature surfactant protein.

The fusion proteins of the invention are generated by means of recombinant DNA technology, which allows complete control of the sequence of an individual fusion protein and thus of its biophysical characteristics. Mutations within the amino acid sequence can be accomplished very easily on DNA level using established standard methods [28].

Possible alterations of the amino acid sequence are insertions or deletions as well as amino acid substitutions. Such substitutions may be conservative, i.e. an amino acid residue is replaced with a chemically similar amino acid residue. Compilations of the properties of amino acid residues are well known in the art. Examples of conservative substitutions are the replacements among the members of the following groups: 1) alanine, serine, and threonine; 2) aspartic acid and glutamic acid; 3) asparagine and glutamine; 4) arginine and lysine; 5) isoleucine, leucine, methionine, and valine; and 6) phenylalanine, tyrosine, and tryptophan.

One the other hand, it is also possible to introduce non-conservative alterations in the amino acid sequence. Since SP-B, for example, is rich in cysteine residues, which form inter- as well as intramolecular disulfide bridges, one such substitution could be the replacement of a cysteine residue with alanine to prevent the formation of disulfide bridges that may interfere with the biophysical and/or catalytic properties of the inventive fusion proteins. Another possible substitution could be the replacement of one or more valine residues of SP-C, e.g., with glycine in order to reduce the hydrophobicity of this protein. However, it is not only possible to change single amino acid residues but also complete domains of the fusion protein according to the invention. For example, portions of the protein that are not involved in catalysis and are not crucial for folding into a functional three-dimensional structure could be removed to reduce the size of the fusion protein, which may be advantageous in many respects.

In general, such modifications of the amino acid sequence are intended to improve the biophysical characteristics and/or the catalytic properties of the inventive fusion protein (e.g., the

half-life *in vivo*, the membrane permeability or its acid resistance in the case of oral administration).

The terms "precursor protein" or "precursor" as used herein refer to a protein that is not completely processed to its mature form but still comprises its N- and/or C-terminal propeptides. The terms "protein component" or "component" refer to the surfactant protein precursors as well as plasminogen activators comprising the fusion proteins of the invention.

In preferred embodiments of the invention, at least one component of the fusion protein as disclosed herein, i.e. the surfactant protein component and the plasminogen activator component, respectively, is a human protein. Most preferred are fusion proteins wherein both components are human proteins (see also Fig. 2).

The invention also includes fusion proteins comprising components, which differ from what is referred to as "wild-type" protein as a result of alternative splicing of a common pre-mRNA molecule, but are nevertheless functional.

The surfactant protein component of the fusion protein may be any known surfactant protein, i.e. surfactant protein SP-A, -B, -C, or -D, with the hydrophobic proteins SP-B and SP-C being preferred, and with SP-B being most preferred. As already outlined above, fibrin formation in the presence of pulmonary surfactant has been shown to result in an almost complete incorporation of these two proteins into the fibrin clot, which makes them suitable candidates for targeting another protein, in this case a plasminogen activator, to surfactant containing clots.

The SP-B precursor (SEQ ID NO: 1) comprises the "mature peptide" (79 amino acids) flanked by a 200 amino acid N-terminal propeptide (including a 23 amino acid signal peptide) and a 102 amino acid C-terminal propeptide, respectively. The fragment comprising the N-terminal propeptide and the mature peptide (as shown in SEQ ID NO: 2) was demonstrated to be necessary and sufficient for both correct folding and transport of SP-B. The removal of the N-terminal propeptide and release of mature SP-B (SEQ ID NO: 3) occurs in type II alveolar cells. So far, it has not been possible to produce mature SP-B in any conventional cell culture systems, such as HeLa cells or CHO cells (cf. above).

Thus, in a preferred embodiment of the invention, the surfactant protein component of the fusion protein is SEQ ID NO: 2.

In an alternative preferred embodiment of the invention, the surfactant protein component of the fusion protein is SEQ ID NO: 3.

The post-translational processing of the SP-C precursor (SEQ ID NO: 8) is very similar to that of SP-B. Mature SP-C (SEQ ID NO: 10), a small protein of only 35 amino acids, is produced by subsequent cleavage of the C- and N-terminal propeptide, respectively (reviewed in [9, 10, 12]).

In another preferred embodiment of the invention, the surfactant protein precursor of the fusion protein is SP-C (as shown in SEQ ID NO: 9).

In a further preferred embodiment, the surfactant protein component of the fusion protein is SEQ ID NO: 10.

A preferred fusion partner for SP-B and SP-C, respectively, with regard to an object of the invention, i.e. lysis of surfactant containing fibrin clots, is urokinase-plasminogen activator (u-PA), since it is the predominant plasminogen activator in the alveolar space. Urokinase-plasminogen activator is synthesized as a 411 amino acid precursor protein as well, which is termed single-chain u-PA (or pro-urokinase; SEQ ID NO: 4). Cleavage between Lys-158 and Ile-159 results in the formation of high molecular weight two-chain u-PA (HMW-u-PA). Further processing by cleavage between Lys-135 and Lys-136 generates low molecular weight two-chain u-PA (LMW-u-PA; SEQ ID NO: 5), which is reported to have a similar enzymatic acivity as the high molecular weight form. The two chains of the protein are connected by a disulfide-bridge between Cys-148 and Cys-279. However, it is possible to use in the present invention any proteinaceous plasminogen activator or fragment or mutant thereof as long as this polypeptidic molecule has plasminogen activator activity.

In a further preferred embodiment of the invention the plasminogen activator of the fusion protein is LMW-u-PA (SEQ ID NO: 5).

Most preferably, the fusion protein of the invention is selected from the group consisting of SEQ ID NO: 6 and SEQ ID NO: 7 comprising chimeras of the SP-B precursor ($SP\text{-}B_{\Delta C}$) and LMW-u-PA, which are referred to as SPUC1A and SPUC1B, respectively (see also Fig. 1A and 1B).

In another particular preferred embodiment of the invention, the fusion protein is selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13 comprising chimeras of the mature

SP-B ($SP\text{-}B_{mature}$) and LMW-u-PA, which are referred to as SPUC2C and SPUC3B, respectively (see also Fig. 1C and 1D).

Also preferred is a fusion protein comprising tissue-plasminogen activator (t-PA; SEQ ID NO: 11) as plasminogen activator component.

Additional non-limiting examples of plasminogen activators suitable for fusion proteins according to the invention are: high molecular weight two-chain u-PA (HMW-u-PA), LMW-u-PA B-chain, recombinant single-chain u-PA (r-scu-PA), recombinant t-PA (rt-PA), and its variants r-PA, n-PA, and TNK-t-PA, desmodus salivary plasminogen activator α-1 (bat-PA), streptokinase, staphylokinase, and catalytically active mutants thereof. Examples of suitable plasminogen activators are also illustrated in Fig. 2.

In a further preferred embodiment of the invention the fusion protein carries a protein or peptide affinity tag at its N-terminus and/or at its C-terminus in order to allow easy detection and/or purification of the recombinant protein. Suitable affinity tags are, for example, the *myc*-tag, the FLAG-tag, the His_6-tag, the Strep-Tag® or the HA-tag.

The present invention also relates to nucleic acid molecules (DNA and RNA) comprising nucleotide sequences coding for fusion proteins as described herein. Since the degeneracy of the genetic code permits substitutions of certain codons by other codons specifying the same amino acid, the invention is not limited to a specific nucleic acid molecule encoding a fusion protein of the invention but includes all nucleic acid molecules comprising nucleotide sequences encoding a functional fusion protein.

The invention also includes nucleic acid molecules encoding a functional fusion protein that comprises nucleic acid sequences different from what is referred to as "wild-type" nucleic acid sequence due to alternative splicing of a common pre-mRNA molecule. Such splicing events include the alternative use of exons (i.e. nucleic acid sequences encoding an amino acid sequence), exon shuffling (i.e. an alternative arrangement of exons), and the retention of introns (i.e. intervening sequences normally not encoding an amino acid sequence) within the mature mRNA molecule.

In preferred embodiments of the invention at least one component of the fusion protein, i.e. the surfactant protein component and the plasminogen activator component, respectively, is encoded

by a human nucleic acid sequence. Most preferred are fusion proteins wherein both components are encoded by human nucleic acid sequences (see also Fig. 2).

In another preferred embodiment the nucleic acid sequence encoding the surfactant protein component of the fusion protein as disclosed herein is selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 9, with the former one preferred.

Also preferred are the nucleic acid sequences encoding a fusion protein as disclosed herein, wherein the surfactant protein component is selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 10, with the former one preferred.

Most preferably, the nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 6 and SEQ ID NO: 7 (see also Fig. 1A and 1B).

In a further particular preferred embodiment of the invention, the nucleic acid molecule comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 12 and SEQ ID NO: 13 (see also Fig. 1C and 1D).

A nucleic acid molecule disclosed in this application may be "operably linked" to a regulatory sequence (or regulatory sequences) to allow expression of this nucleic acid molecule.

A nucleic acid molecule, such as DNA, is referred to as "capable of expressing a nucleic acid molecule" or capable "to allow expression of a nucleotide sequence" if it comprises sequence elements which contain information regarding to transcriptional and/or translational regulation, and such sequences are "operably linked" to the nucleotide sequence encoding the polypeptide. An operable linkage is a linkage in which the regulatory sequence elements and the sequence to be expressed are connected in a way that enables gene expression. The precise nature of the regulatory regions necessary for gene expression may vary among species, but in general these regions comprise a promoter which, in prokaryotes, contains both the promoter *per se*, i.e. DNA elements directing the initiation of transcription, as well as DNA elements which, when transcribed into RNA, will signal the initiation of translation. Such promoter regions normally include 5' non-coding sequences involved in initiation of transcription and translation, such as the -35/-10 boxes and the Shine-Dalgarno element in prokaryotes or the TATA box, CAAT sequences, and 5'-capping elements in eukaryotes. These regions can also include enhancer or

repressor elements as well as translated signal and leader sequences for targeting the native polypeptide to a specific compartment of a host cell.

In addition, the 3' non-coding sequences may contain regulatory elements involved in transcriptional termination, polyadenylation or the like. If, however, these termination sequences are not satisfactory functional in a particular host cell, then they may be substituted with signals functional in that cell.

Therefore, a nucleic acid molecule of the invention can include a regulatory sequence, preferably a promoter sequence. In another preferred embodiment, a nucleic acid molecule of the invention comprises a promoter sequence and a transcriptional termination sequence. Suitable prokaryotic promoters are, for example, the *lac*UV5 promoter or the T7 promoter. Examples of promoters useful for expression in eukaryotic cells are the SV40 promoter or the CMV promoter.

The nucleic acid molecules of the invention can also be comprised in a vector or other cloning vehicles, such as plasmids, phagemids, phage, baculovirus, cosmids or artificial chromosomes. In a preferred embodiment, the nucleic acid molecule is comprised in a vector, particularly in an expression vector. Such an expression vector can include, aside from the regulatory sequences described above and a nucleic acid sequence encoding a fusion protein of the invention, replication and control sequences derived from a species compatible with the host that is used for expression as well as selection markers conferring a selectable phenotype on transformed or transfected cells. Most preferably, the nucleic acid molecule is comprised in an expression vector adapted for expression of a eukaryotic coding sequence. Large numbers of suitable vectors are known in the art, and are commercially available.

The DNA molecule encoding fusion proteins of the invention, and in particular a vector containing the coding sequence of such a fusion protein can be transformed into a host cell capable of expressing the gene. Transformation can be performed using standard techniques [28]. Thus, the invention is also directed to a host cell containing a nucleic acid molecule as disclosed herein.

The transformed host cells are cultured under conditions suitable for expression of the nucleotide sequence encoding a fusion protein of the invention. Suitable host cells can be prokaryotic, such as *Escherichia coli* (*E. coli*) or *Bacillus subtilis*, or eukaryotic, such as *Saccharomyces cerevisiae*,

Pichia pastoris, SF9 or High5 insect cells, immortalized mammalian cell lines (e.g. HeLa cells or CHO cells), primary mammalian cells or pulmonary stem cells.

The invention also relates to a method for recombinant production of fusion proteins according to the invention. This method comprises:

(a) introducing a nucleic acid molecule encoding the fusion protein into a suitable vector, and

(b) introducing the recombinant vector obtained in (a) into a suitable host cell or into a suitable cell extract.

Step (a) can be performed with a nucleic acid molecule encoding only the fusion protein. Alternatively, it can be performed with a nucleic acid molecule in which the fusion protein coding sequence is operably linked to regulatory sequences. Optionally, the nucleic acid molecule of the invention can also be fused to a sequence coding for a fusion partner such as an affinity tag allowing easy detection and/or purification of the recombinant fusion protein. In another embodiment of the method of the invention, the nucleic acid sequences encoding the surfactant protein and the plasminogen activator component, respectively, of the fusion protein as disclosed herein may be independently from each other inserted into a suitable vector. Gene expression can be achieved in a recombinant cell or a suitable cell extract, which contains all factors required for transcription and translation.

Furthermore, the present invention refers to pharmaceutical uses of the inventive fusion protein. In one embodiment, the invention refers to a method for prophylaxis and/or treatment of inflammatory and interstitial lung diseases, comprising the step of administering a fusion protein as disclosed herein alone or in combination with other pharmaceutically active compounds and a pharmaceutically acceptable excipient to a mammal, and in particular to a human.

Acute or chronic inflammatory and interstitial lung diseases or lung disorders which may be prevented or treated with a fusion protein described in this application include the acute (or adult) respiratory distress syndrome (ARDS), acute lung injury (ALI), interstitial lung disease (ILD), idiopathic pulmonary fibrosis (IPF), sarcoidosis, hypersensitivity pneumonitis, pulmonary inflammation, pneumonia, bronchitis, asthma, cystic fibrosis, surfactant abnormalities in recurrent apparent life-threatening events (ALTE) or the sudden infant death syndrome (SIDS), congenital alveolar proteinosis and the severe acute respiratory syndrome (SARS).

The fusion proteins according to the invention can be administered via any parenteral, non-parenteral (enteral) or topical (intratracheal) route that is therapeutically effective for proteinaceous drugs. Parenteral application methods comprise, for example, intracutaneous, subcutaneous, intramuscular or intravenous injection and infusion techniques, e.g. in the form of injection solutions, infusion solutions or tinctures, as well as aerosol installation and inhalation, e.g. in the form of aerosol mixtures, sprays or dry powders. Non-parenteral delivery modes are, for instance, orally, e.g. in the form of pills, tablets, capsules, solutions or suspensions, or rectally, e.g. in the form of suppositories. The fusion proteins of the invention can be administered systemically or topically in formulations containing conventional non-toxic pharmaceutically acceptable excipients or carriers, additives and vehicles as desired.

In a preferred embodiment of the present invention the fusion protein is administered parenterally to a mammal, and in particular to humans, with aerosol administration or intratracheal installation being the most preferable application method.

The dosage of the fusion protein of the present invention may vary within wide limits to achieve the desired therapeutic response for a particular patient. It will, for instance, depend on the enzymatic, i.e. fibrinolytic, activity of the fusion protein as well as its half-life *in vivo*, the mode of administration, the severity of the disease/disorder being treated, as well as the medical condition of the patient. For example, treatment of acute short-term disorders, such as an asthmatic attack or acute lung injury, might be best accomplished when using a dose as high as maintainable. In contrast, for treatment of long-term chronic disorders, such as interstitial lung disease or idiopathic pulmonary fibrosis, a lower dosage, optionally given in a sustained release formulation, might be more suitable. The establishment of a therapeutically effective dosage amount for a given individual is within the level of skill in the art.

In general, a daily dose of about 500 μg to 200 mg fusion protein per kilogram body weight may be appropriate. Preferred dosage levels range from 0.5 mg to 50 mg/kg body weight/day for a long-term regimen and from 50 mg to 200 mg/kg body weight/day for short-term treatments. The fusion protein can be applied as a single dose or may be divided into several, e.g., two to four, part administrations.

Thus, the invention is also directed to a pharmaceutical composition comprising a fusion protein as described above and a pharmaceutically acceptable excipient. In particular, the invention refers to a pharmaceutical composition, which has fibrinolytic activity.

Recombinant fusion proteins of the invention can be formulated into compositions using pharmaceutically acceptable ingredients as well as established methods of preparation [29]. To prepare the pharmaceutical compositions, pharmaceutically inert inorganic or organic excipients can be used. To prepare e.g. pills, powders, gelatin capsules or suppositories, for example, lactose, talc, stearic acid and its salts, fats, waxes, solid or liquid polyols, natural and hardened oils. Suitable excipients for the production of solutions, suspensions, emulsions, aerosol mixtures or powders for reconstitution into solutions or aerosol mixtures prior to use include water, alcohols, glycerol, polyols, and suitable mixtures thereof as well as vegetable oils.

The pharmaceutical composition may also contain additives, such as, for example, fillers, binders, wetting agents, glidants, stabilizers, preservatives, emulsifiers, and furthermore solvents or solubilizers or agents for achieving a depot effect. The latter is that fusion proteins may be incorporated into slow or sustained release or targeted delivery systems, such as liposomes, nanoparticles, and microcapsules.

For example, fusion proteins of the invention can be admixed to commercially available surfactant preparations and administered by aerosol administration or transbronchial instillation or via a bronchoscope. Suitable surfactant preparations include, e.g., Survanta® as well as Alveofact®, two natural bovine surfactant preparations, Infasurf®, a calf-lung surfactant extract, and Exosurf®, a synthetic surfactant composition lacking the hydrophobic proteins SP-B and SP-C.

The invention is further illustrated by the following non-limiting Figures and Examples.

Figure 1 shows schematic representations of four exemplary expression vectors according to the invention. The vectors illustrated in Fig. 1A and 1B encode fusion proteins consisting of SP-$B_{\Delta C}$ N-terminally fused to LMW-u-PA (SEQ ID NO: 6 and SEQ ID NO: 7, respectively). pSPUC1A (Fig. 1A) is derived of pcDNA3.1(-) (Invitrogen), whereas pSPUC1B (Fig. 1B) is derived of pSecTag2A (Invitrogen). Figure 1C illustrates pSPUC2C encoding a fusion protein composed of SP-B_{mature} N-terminally fused to LMW-u-PA (SEQ ID NO: 12), wherein this gene fusion is preceeded by a segment encoding the SP-B signal peptide as well as a 6-nucleotide spacer element. Figure 1D depicts vector pSPUC3B encoding a fusion protein consisting of SP-B_{mature} C-terminally fused to LMW-u-PA (SEQ ID NO: 13),

wherein the LMW-u-PA cDNA is preceeded by a segment encoding the u-PA signal peptide as well as a 6-nucleotide spacer element

Figure 2 schematically illustrates the design of a fusion protein according to the invention. A mammalian surfactant protein component is fused at its C-terminus to the N-terminus of a mammalian plasminogen activator. Either one of these components or both can be human proteins. The two protein components can be selected from the non-limiting examples indicated at the bottom part of the Figure. Importantly, if the surfactant protein component is a mature surfactant protein, it is also within the scope of the invention that the mature surfactant protein can be fused with its N-terminus to the C-terminus of a plasminogen activator.

Figure 3 documents successful expression of recombinant SPUC1A (SEQ ID NO: 6) in Chinese hamster ovary (CHO) cells. 35 hours following transfection with pSPUC, cells were harvested and labeled with [^{35}S]-methionine/cysteine for 6 hours. Supernatants (S) and cell lysates (C) were immunoprecipitated with the antibodies indicated, and bound proteins were separated by SDS-PAGE. Signals were visualized by autoradiography. A fusion protein of correct size (about 65 kDa) could be concordantly detected with antibodies specific for both components of the protein, respectively.

Figure 4 depicts the amidolytic activity of SPUC1A (SEQ ID NO: 6) compared to full-length SP-B (SP-B_{FL}; SEQ ID NO: 1) and LMW-u-PA (SEQ ID NO: 5) in CHO cells: Cell samples were harvested either 20 hours (supernatants, S) or 44 hours (supernatants, S and cell lysates, C) after transfection with the respective DNAs, transferred to microtiter plates and incubated with the chromogenic substrates, Chromozyme U (direct substrate for u-PA) and S-2251 (indirect substrate, addition of plasminogen required), respectively. The absorbance (405 nm) of the samples was determined in a microplate reader. Cells transfected with pSPUC1A exhibited amidolytic activity, which was more pronounced after the addition of plasminogen. Thus, recombinant SPUC is functional when expressed in CHO cells.

Example 1: Cloning of SPUC1A cDNA

The vector pSPUC1A (Fig. 1A) encoding a fusion protein (termed SPUC1A; SEQ ID NO: 6) consisting of human SP-$B_{\Delta C}$ (SEQ ID NO: 2) N-terminally fused to human low molecular weight

urokinase-plasminogen activator (LMW-u-PA; SEQ ID NO: 5) was constructed using standard methods [28]. The respective cDNA fragments were inserted into the multiple cloning site of the expression vector pcDNA3.1(-) (Invitrogen) under control of the CMV promoter. The SP-$B_{\Delta C}$ cDNA was cloned between the XhoI and HindIII sites of the multiple cloning site, and the LMW-u-PA cDNA between the HindIII and AflII sites.

The ligation mixture obtained was transformed into *E. coli*, and single clones were screened for presence of the correct insert by PCR analysis using primers flanking the site of insertion. Positive transformants were amplified in *E. coli*. The vector-DNA was purified by ion-exchange chromatography and sequenced using an automated system (ABI Prism 310 Genetic Analyzer; Perkin Elmer).

Example 2: Expression of SPUC1A in CHO cells

Chinese hamster ovary (CHO) cells (American Type Culture Collection) were grown as monolayers at 37°C and 10% CO_2. Growth medium consisted of a 1:1 mixture of DMEM and DMEM-F12 supplemented with 10% fetal calf serum, 20mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. DNA transfection was performed using 2.5 µg pSPUC1A and Lipofectamine Plus (Life Technologies/GIBCO BRL) according to the instructions of the manufacturer.

The expression of SPUC1A (SEQ ID NO: 6) was analyzed using [^{35}S] cell labeling and immunoprecipitation (Fig. 3). 35 h following transfection, the growth medium was replaced with DMEM supplemented with 10% FCS and 25mM HEPES, but free of methionine/cysteine. After a 40 min incubation period, the cells were labeled for 6 h with 0.5 mCi/ml [^{35}S]-methionine/cysteine (Pro-mix [^{35}S] *in vitro* cell labeling mix; Amersham).

Supernatants (S) as well as cell lysates (C) were then immunoprecipitated with a polyclonal rabbit anti-human pro-SP-B antibody (Chemicon) and a monoclonal mouse anti-human u-PA antibody (American Diagnostica), respectively. Protein G-Sepharose (30 µl; Zymed Laboratories) and rabbit serum (5 µl) were added to each tube and the samples incubated on a rotator at 4°C for 12h. After centrifugation at 1.000 x g, the supernatants were transferred to new tubes, and 30 µl Protein-G-Sepharose and 5 µl of the respective antibody were added. After another incubation period (12 h, 4°C) and subsequent centrifugation, the pellets were washed four times with wash buffer A (150mM NaCl, 50mM Tris, 5mM EDTA, 0.1% Triton X-100, 0.02% SDS, pH 7.6) and twice with wash buffer B (150mM NaCl, 50mM Tris, 5mM EDTA, pH 7.6). The samples were

suspended in Laemmli-buffer, boiled for 5 min, and run on a 10% SDS-PAGE gel. The gel was fixed for 1 h in 40% methanol/10% glacial acid/4% glycerol, incubated for 30 min in enhancer solution, dried in a vacuum chamber, and exposed to an X-ray film (Kodak Biomax MR).

A fusion protein of the expected size (about 65 kDa) could be concordantly detected with antibodies specific for both components of the protein, respectively (Fig. 3). Thus, recombinant SPUC1A can be successfully expressed in CHO cells. A preliminary quantification of SPUC1A levels by ELISA analysis using the monoclonal mouse anti-human u-PA antibody (data not shown) resulted in concentrations ranging from 34 to 58 ng/ml supernatant.

Example 3: Functional analysis of SPUC1A using chromogenic substrates

The amidolytic activity of recombinant SPUC1A (SEQ ID NO: 6) in CHO cell supernatants and lysates was determined using the chromogenic substrates Chromozyme U (Roche Diagnostics) and S-2251 (Chromogenix), respectively. The assay buffer consisted of 100mM Tris, pH 7.6, 0.5% Tween-20, and 100 µg/ml BSA.

Chromozyme U is a direct substrate for u-PA. Test samples (cell supernatants 20 and 44 h after transfection as well as cell lysates) were transferred in a volume of 50 µl to a microtiter plate and incubated with 100 µl assay buffer and 100 µl Chromozyme U (1 mg/ml). Reactions were terminated by addition of 50 µl acetic acid (50% solution), and the absorbance was determined at 405 nm. S-2251, on the other hand, is an indirect substrate for u-PA that is cleaved after activation of plasminogen to plasmin. Test samples were also transferred to a microtiter plate and mixed with 100 µl of a diluted plasminogen solution (50 µg/ml) and 100 µl S-2251 (2mM) dissolved in assay buffer. After incubation, reactions were terminated by addition of 50 µl acetic acid, and the absorbance at 405 nm was measured. Cells transfected with $pSPB_{FL}$ encoding human full-length SP-B (SEQ ID NO: 1) served as negative control, whereas cells transfected with pLMW-u-PA encoding human LMW urokinase-plasminogen activator (SEQ ID NO: 5) served as positive control.

After transfection of CHO cells with pSPUC1A, amidolytic activity could be detected in the cell supernatants (Fig. 4). However, the effect was more pronounced after addition of plasminogen when using S-2251 as a substrate. In cells transfected with $pSPB_{FL}$ no measurable amidolytic activity was observed, as expected. Cells transfected with pLMW-u-PA showed much higher levels of u-PA activity compared to cells transfected with pSPUC1A. The reason for this finding

remains unclear and has to be addressed in further studies. Nevertheless, these results confirmed that recombinant SPUC1A is indeed functional when heterogeneously expressed in CHO cells.

Example 4: Functional analysis of SPUC1A by fibrin gel autography

As a second measure of plasminogen activator activity CHO cell supernatants and lysates were analyzed by fibrin gel autography, which was performed as described [30]. The samples were separated via SDS-PAGE using 10% acryl-amide resolving gels. The gel was soaked for 1.5 h in 0.1 M sodium phosphate pH 7.2 with 5% Triton X-100 to neutralize SDS and then placed on top of a fibrin indicator gel. In brief, a 2% (w/v) agarose solution was boiled, cooled to 45°C and mixed with pre-warmed phosphate-buffered saline containing 140 μg/ml plasminogen and 0.8 U/ml thrombin. Fibrinogen (10 mg/ml) in PBS (37°C) was added and the mixture was poured onto a glass plate. Final concentrations were 1% agarose, 35 μg/ml plasminogen, 0.2 U/ml thrombin, and 2 mg/ml fibrinogen. The fibrin gel was developed in a moist chamber and photographed. Plasminogen activators were revealed by formation of dark lytic zones in the opaque fibrin matrix of the indicator gel.

In CHO cells transfected with pSPUC1A, a lytic zone migrating at about 65 kDa could be identified in both supernatants and cell lysates (data not shown). This finding is in full agreement with the results obtained in the cell labeling/immunoprecipitation studies (Fig. 3; Example 2) as well as the cleavage experiments (Fig. 4; Example 3) described above, further substantiating the functionality of recombinant SPUC1A.

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